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16. ABSTRACT

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4. The ratio of acidic to neutral glycoproteins secreted by tracheal explants varied with duration of incubation of the trachea in culture.

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COMPOSITION OF GLYCOPROTEINS SECRETED BY TRACHEAL EXPLANTS FROM VARIOUS ANIMAL SPECIES

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Abstract—1. The acidic and neutral glycoproteins secreted by cultured tracheal explants from pigs, sheep, rats, mice, monkeys, guinea pigs, dogs and chickens were purified and fractionated by column chromatography on DEAE-cellulose and by electrophoresis on cellulose acetate.

2. The ratios of acidic to neutral mucus glycoproteins were compared for the above animals with that of mucus glycoproteins secreted by cultured human bronchi.

3. The observed ratios of acidic to neutral glycoproteins ranged from 4.0 (mouse) to 7.2 (chicken and pig) from cultured tracheae; secreted human bronchial mucus had a ratio of 2.7.

4. The ratio of acidic to neutral glycoproteins secreted by tracheal explants varied with duration of incubation of the trachea in culture.

INTRODUCTION

Tracheobronchial mucus is a principal component of the mucociliary system which plays an important role in the defense of the lung. The efficiency of the mucociliary clearance apparatus is due to the chemical and physical properties of mucus and to the propelling ciliary function. In pathophysiological conditions such as in toxic inhalant exposures, over-production of mucus and/or changes in its composition may perhaps alter overall lung defense capabilities, either by enhancing defenses or by contributing to the development of chronic obstructive airflow disease.

Little is known about differences in the chemical composition of respiratory system mucus from various species. Analyses of the carbohydrate and amino acid composition of partially purified glycoproteins prepared from sputum from humans (Boat & Mathews, 1973; Roussel *et al.*, 1975) and nonhuman primates (Hatcher *et al.*, 1977) and mucus collected from tracheal pouches in dogs (Chakrin *et al.*, 1972) have been published. Few, if any, comparative studies of tracheal mucus among different species have been published of which we are aware. Meyer (1977) has compared the buoyant density and sugar content of bovine cervical, human tracheal and middle ear, and frog palatal mucus; he found the mammalian glycoproteins to be very similar, while the amphibian glycoproteins had a lower sugar content and, hence, a lower buoyant density. While as a generalization it is true that all species examined produce both neutral and acidic mucus glycoproteins, as is known from histochemical studies (Chakrin *et al.*, 1972; Reid, 1968), the relative amounts of neutral and acidic components have not been systematically compared among different species, nor is the chemical basis for "acidic" and "neutral" glycoprotein components known to be exactly the same among different animals examined.

In an earlier paper (Jennings *et al.*, 1977) we compared the rates of biosynthesis and secretion of mucus glycoproteins by tracheal explants from seven mammalian species. The similarity of the measured relative rates of secretion was a striking finding. In the present paper, we present a comparative study of the nature of the glycoproteins secreted by the various tracheal explants earlier reported upon, as well as additional data on the glycoproteins secreted by tracheae from a nonmammalian species, the chicken, and by bronchial explants from humans.

MATERIALS AND METHODS

Animals

All large animals used in these studies were obtained from the University of California farms; rats were chronic respiratory disease-free (Hilltop, Scottsdale, PA) and guinea pigs were specific pathogen-free.

Tracheal and bronchial explants

Explants were prepared and cultured in the presence of radioactive D-glucosamine exactly as described previously (Jennings *et al.*, 1977). Chicken (*Gallus gallus*) tracheae were prepared as was described for small animals. Human (*Homo sapiens*) bronchi were prepared as described for large animals. Acid-precipitable radioactivity was determined as we described earlier (Jennings *et al.*, 1977).

The labelled glycoproteins in the tracheal culture media from the different species were further characterized by purification and fractionation by chromatography and/or electrophoresis, using the methods detailed below.

Papain digestion

Crystalline papain (3 mg/ml of final incubation medium, Sigma Chemical Co.) was added to 0.1 M citrate buffer, pH 5.6, containing 0.1 M NaCl, 5 mM EDTA, and 1 mM cysteine. The enzyme was activated by being heated at 60°C (Scott, 1960). Dialyzed tracheal explant media (see above) were mixed with 0.1 volume of a 10-fold concentrate of this solution and incubated at 60°C for 24 hr. Ad-

ditional crystalline papain (3 mg/ml each time) was added to the incubation mixture after 6 and 22 hr. After the solution was cooled to 4°C, protein was precipitated by the addition of trichloroacetic acid to a final concentration of 5% (w/v). The mixture was left at 4°C for 2 hr, then the precipitate was separated by centrifugation at 10,000 rev/min (Sorvall, SS rotor) for 20 min. After a single extraction with cold 5% trichloroacetic acid, the precipitate was discarded and the supernatant was cooled and dialyzed exhaustively against water, then concentrated by lyophilization for subsequent column chromatography.

DEAE-cellulose chromatography

DEAE-cellulose (Whatman, DE-52) was equilibrated with 0.01 M LiCl in 0.05 M acetate buffer, pH 4.4. Trichloroacetic acid-soluble fractions were dissolved in 500 μl of distilled water. Columns (0.9 × 3 cm) were prepared and samples (400 μl) were applied in water; 50 μl aliquots were also counted to allow calculation of recoveries from the columns. The neutral glycoprotein fraction, eluted with 0.05 M acetate buffer, passed through the DEAE-cellulose without binding, and was collected as a 10 ml pool. The eluting solvent was then changed to 1 M LiCl in the same acetate buffer, and a second pool of 10 ml was collected. A 500 μl aliquot of each pool was counted by liquid scintillation in 9 ml of Scintiverse (Fisher Chemical Co.). The remainder of each fraction was dialyzed against several changes of water, then lyophilized to dryness.

Alternatively, the acidic fraction(s) could be eluted from DEAE-cellulose with a linear salt gradient, as detailed in the legend to Fig. 1.

Alkaline borohydride reduction

Fractions from the DEAE-cellulose column were treated with 0.05 M NaOH–2.0 M $NaBH_4$ for 18 hr at 45°C in a final volume of 30 ml (Hatcher *et al.*, 1977). The excess of borohydride was eliminated by addition of acetic acid at 4°C. The sample was desalted with AG50W-X8 (H^+, 100–200 mesh, Bio-Rad) cation-exchange resin in a 1 × 10 cm column at room temperature; the resultant aqueous solution was neutralized by the addition of pyridine and lyophilized. Alternatively, the sample was desalted on a column (1.0 × 58 cm) of Biogel P-2 (100–200 mesh) or dialyzed exhaustively against distilled water, and the resultant salt-free solution was lyophilized.

Electrophoresis on cellulose acetate

Fractions from DEAE-cellulose chromatography and alkaline borohydride-treated fractions were examined by zone electrophoresis on cellulose acetate strips in a Beckman Microzone electrophoresis apparatus containing barbital buffer, 0.075 ionic strength and pH 8.6 (Beckman). Buffer-soaked strips, with excess moisture removed by gentle blotting, were placed in the apparatus. Up to 20 μl of solution was applied to the strip in a thin line; a current of 0.48 mA/cm was then applied for about 6 min. Strips were cut into pieces of 0.5 cm each and counted for radioactivity by liquid scintillation in 10 ml of counting cocktail (Scintiverse).

RESULTS

In Table 1 we demonstrate the essential equivalence of tracheal and bronchial explants in our culture system by a comparison of the rate of secretion by similar sized pieces from these two areas of conducting airway from rhesus monkeys. Tracheal and bronchial slices of the same size reproducibly secrete equivalent amounts of labelled mucus glycoproteins in organ culture, whether glucosamine or fucose is used as the radioactive precursor. Human bronchial explants were, therefore, included in this study as such material was available from lung resection operations performed for therapeutic purposes.

After incubation of tracheae or bronchi, samples were processed prior to ion-exchange chromatography as described in Methods. The treated culture medium was fractionated on columns of DEAE-cellulose by gradient elution techniques. A typical separation of neutral and acidic glycoproteins from rat tracheal explants is illustrated in Fig. 1(a). We have shown elsewhere (Kaizu & Last, 1978) that the acidic glycoproteins in peak C may be converted to material with identical mobility to that of peak B by treatment with bacterial neuraminidase. The neutral glycoprotein fraction, peak A, is not retained by DEAE-cellulose at pH 4.4 and passes through the column during loading of samples. The characteristic profile of mucus glycoproteins secreted by rat tracheal explants may be compared with those secreted by human bronchial explants incubated under the same culture conditions, as shown in Fig. 1(b). Peaks with the same mobility as A, B and C from rat tracheal explants are again observed, as well as a more acidic peak, called "D" (which is eluted prior to authentic chondroitin-4-sulfate; see below). The relative ratio of counts contained in peak B to those in peak C from rat tracheal explants is, in part, a function of sample handling due to the lability of terminal sialic acid residues below pH values of about 5

Table 1. Comparison of glycoprotein secretion rates by rhesus monkey tracheal and bronchial explants

Experiment Number	Hours Incubated	Precursor Used	CPM of labeled glycoprotein secreted	
			Trachea	Bronchi
1	23	[^{14}C]glucosamine	8179 ± 954(4)	6864 ± 1585(4)
2	23	[^{14}C]glucosamine	9081 ± 1221(4)	11,866 ± 3008(4)
3	18	[^{3}H]fucose	1147 ± 150(5)	922 ± 196(5)

Data are presented as mean counts/min secreted ±S.D. Values of *n*, the number of slices (explants) evaluated, are given in parentheses. In experiments 1 and 2, the precursor used was [1-^{14}C]D-glucosamine (56.6 Ci/mol, 1.25 μCi/ml); in Experiment 3 the precursor used was [1, 5, 6-^{3}H]L-fucose (2.4 Ci/mmol, 2.5 μCi/ml).

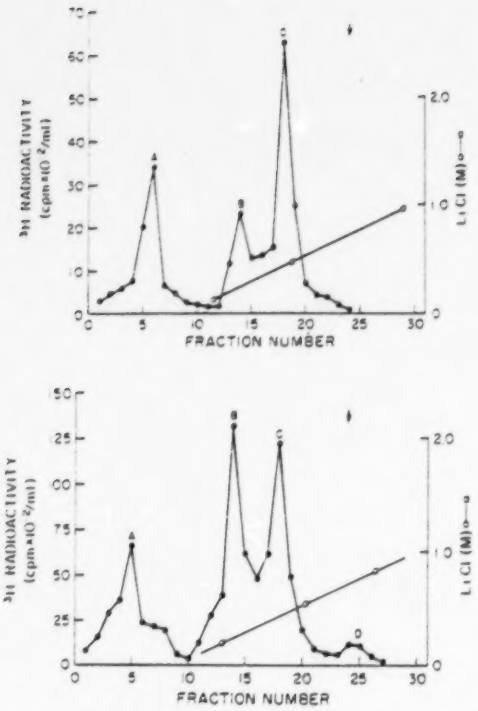

Fig. 1. (a) Separation of glycoproteins secreted by rat tracheal explants, incubated for 24 hr in culture with [^{3}H]D-glucosamine, by gradient elution from a DEAE-cellulose column. Medium glycoproteins were treated with papain as described in Methods, then lyophilized. The resultant material was dissolved in 10 mM LiCl–50 mM acetate buffer, pH 4.4, and dialyzed against the same buffer before being loaded on the DEAE-cellulose column (0.9 × 3 cm). Neutral glycoproteins were not bound and passed through the columns (peak "A"). Acidic glycoproteins were eluted with a linear salt gradient (made in a Buchler gradient former, 30 × 30 ml) of LiCl from 0.01 to 2.0 M in 50 mM acetate buffer, pH 4.4 (Hallen, 1972). The column flow rate was 60 ml/hr and 3-ml fractions were collected. The radioactivity in each fraction was evaluated by counting a suitable aliquot in 10 ml of Scintiverse solvent. The elution position of authentic chondroitin-4-sulfate added as an internal standard is at fraction 28. (b) Separation of glycoproteins secreted by human bronchial explants, incubated for 24 hours in culture, by gradient elution from a DEAE-cellulose column.

(Pigman, 1977), conditions such as occur during the DEAE-cellulose column chromatography step (at pH 4.4).

Gradient elution from DEAE-cellulose columns is a tedious procedure for evaluation of large numbers of samples. Thus, we turned to a batchwise elution procedure for analysis of the neutral and total acidic glycoprotein fractions from the culture medium of tracheal explants from the various animals to be compared in this study. The ratio of acidic to neutral glycoproteins in samples of culture medium was determined as: (total counts/min eluted by 0.67 M LiCl)/(total counts/min passing through the column unbound). The observed ratio of acidic to neutral glycoproteins varied from values of about 3 for humans to values of about 7 for pig, chicken, and sheep, as shown in Table 2. Rats and mice had ratios of about 4 (80% acidic glycoproteins, 20% neutral), while monkeys, guinea pigs, and dogs had slightly higher values.

The composition of the secreted mucus glycoproteins from the animal tracheae studied in Table 2 was further investigated by electrophoresis on cellulose acetate strips (Kaizu & Last, 1978). This technique allowed us to determine the relative concentrations of neutral, peak B and peak C glycoproteins (as defined by their mobility on DEAE-cellulose, cf. Fig. 1a) in a sample, although unlike gradient elution from DEAE-cellulose, yields are not quantitative and only relative amounts of the different fractions can be determined. As shown in Fig. 2, two types of composition were observed. One group, comprised of pig, monkey and sheep, had the majority of their glycoprotein coelectrophoresing with the peak B glycoprotein fraction. The other group, consisting of mouse, guinea pig, dog, rat and chicken, had about half of their glycoproteins coelectrophoresing with the neutral glycoprotein fraction. Only chicken contained a substantial component (about 20%) coelectrophoresing with the peak C glycoprotein fraction, while dog and monkey medium contained about 10% of the peak C glycoprotein by this assay.

The correlation between acidic to neutral glycoprotein ratios in Table 2 and those that may be calculated from the data of Fig. 2 is fairly good for pig

Table 2. Ratio of acidic to neutral mucus glycoproteins secreted by tracheal explants from various animals

Animal	Hours Tracheal Explant Incubated	Acidic to Neutral Glycoprotein Ratio
Pig (*Sus scrofa*)	27	7.2
Sheep (*Ovis aries*)	24	6.8 ± 0.5(2)
Rat (*Rattus norvegicus*)	24	4.2 ± 0.7(16)
Monkey (*Macaca mulatta*)	25	5.4
Guinea pig (*Cavia porcellus*)	20	5.1 ± 0.4(3)
Mouse (*Mus musculis*)	24	4.0
Dog (*Canis familiaris*)	24	5.7 ± 0.6(3)
Chicken (*Gallus gallus*)	24	7.2 ± 0.6(2)
Human (bronchial explants) (*Homo sapiens*)	24	2.7

Data are individual chromatographic determinations from single or pooled groups of tracheal explants, or are presented as mean ± S.D. of multiple determinations (values of *n* are given in parentheses). [^{14}C]D-glucosamine was used as the labelled precursor in all of the experiments tabulated except with human, rat and chicken explants where [^{3}H]D-glucosamine was used. Yields of applied glycoproteins recovered from the DEAE-cellulose columns ranged from 91 to 104%.

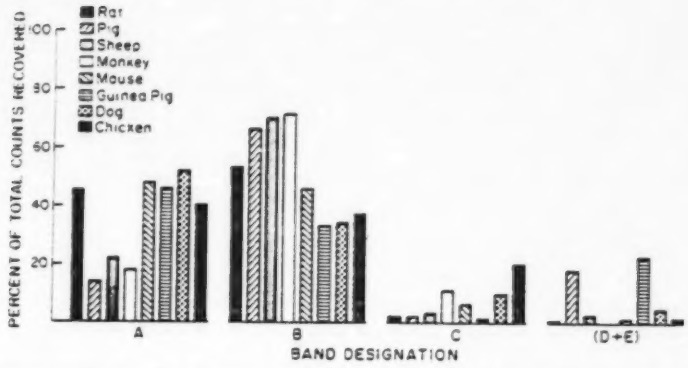

Fig. 2. Relative concentrations of neutral and acidic (peak B and peak C glycoproteins) mucus glycoproteins determined by electrophoresis on cellulose acetate strips. Electrophoresis was performed at 7 mA (0.48 mA.cm) for 6 min on strips soaked in barbital buffer, pH 8.6, 0.075 ionic strength. Radioactivity from labelled glycoproteins was determined by counting 0.5 cm strips by liquid scintillation.

and monkey; however, there seems to be proportionately too much neutral glycoprotein in the cellulose acetate fractions of mouse, guinea pig, sheep, dog and chicken to account for the ratios observed in Table 2. This apparent inconsistency emphasizes the difficulties in trying to use cellulose acetate electrophoresis as a quantitative technique, even though it is very useful for rapidly surveying large numbers of samples in a semi-quantitative way. Of the eight animals whose tracheal explants were evaluated in Fig. 2, sheep, pig, guinea pig, mouse and chicken all contained components more acidic than peak C glycoproteins (presumably glycosaminoglycans; see below) that were missed when the electrophoresis was performed for 12 min. In the cases of pig and guinea pig, the most acidic component (peaks D + E) constituted about 20% of the total cpm recovered upon electrophoresis.

We have previously shown (Kaizu & Last, 1978; Last *et al.*, 1977) that there is no detectable secretion of labelled proteoglycans by rat tracheal explants incubated for 24 hr under the culture conditions used in the present study. The presence of components more acidic than mucus glycoproteins in the culture medium from incubation of mouse, guinea pig and chicken tracheae suggested that explants from these animals might be secreting labelled proteoglycans. We thus looked for labelled glycosaminoglycans that would have been released from proteoglycans by digestion with papain. We examined these materials by gradient elution from DEAE-cellulose. Acidic peaks of labelled material were observed that cochromatographed with authentic chondroitin-4-sulfate (peak "D") and in the approximate elution position expected for dermatan or keratan sulfate (peak "E") when culture medium from chicken tracheal explants was analyzed (Fig. 3). Similar results (data not shown) were observed with medium from guinea pig explants. Only peaks "A", "B", "C" and "D" were present in the culture medium from human tracheal explants (Fig. 1b).

The duration of incubation of tracheal explants in culture affected the relative concentration of acidic mucus glycoproteins present in the medium, as shown in Table 3. In general, the ratio of acidic to neutral glycoproteins in the medium increased with longer incubation times. We have not systematically investigated the nature of the apparent relative increase in the acidic glycoprotein fraction. However, electrophoretic analyses on cellulose acetate strips (data not shown) suggest that there may be augmented secretion of glycoproteins in the moderately acidic class, the peak B glycoprotein fraction. This finding is especially noticeable with pig, monkey and dog tracheal explants.

DISCUSSION

The most striking result of the comparisons presented in this paper, as in our earlier study, seems to be the inherent similarity between glycoproteins secreted by tracheal explants from a large variety of animals. Not only are the rates of secretion and of synthesis very similar (Jennings *et al.*, 1977), but the present study shows the composition of the secreted mucus under standardized incubation conditions, insofar as the ratios of neutral to acidic components are concerned, also to be very similar. The ratio of acidic to neutral glycoproteins ranged from 4.0

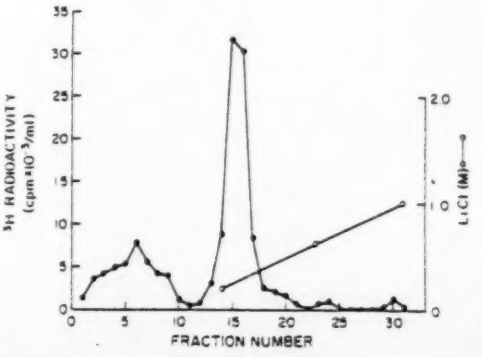

Fig. 3. Separation of labelled glycoproteins secreted by chicken tracheal explants, incubated for 24 hr in culture, by gradient elution from a DEAE-cellulose column. Column and other experimental conditions identical to those of Fig. 1.

Table 3. Effect of duration of incubation of tracheal explants on the ratio of acidic neutral glycoproteins secreted into the culture medium

Animal tested	Hours trachease incubated	Acidic/neutral glycoprotein ratio
Pig	8	2.5
	18	5.3
	27	7.2
Monkey	25	5.4
	69	7.0
Mouse	24	4.0
	72	4.7
Dog*	19	5.4
	44	5.0
	103	8.4

Ratios of acidic to neutral glycoproteins were determined as in Table 2. [^{14}C]D-glucosamine was used to label the mucus glycoproteins in all of the experiments tabulated.

* These results are from a different experiment than the one that generated the data within Table 2.

(mouse) to 7.2 (chicken and pig) among the various experimental animals tested (Table 2). In fact, greater differences in the composition of secreted mucus glycoproteins could often be observed within a given species when duration of incubation of tracheal slices (Table 3) or age of animals used as trachea donors (data not shown) was varied than the differences in composition observed among the interspecies comparisons. Although comparisons between species were based upon data obtained from incubations of from 20 to 27 hr, tracheae from seven of the nine species examined (Table 2) were incubated for 24–25 hr; thus, these variations in incubation time should not have been great enough to prevent valid inferences to be drawn from these data.

All of the animals used in the studies presented herein were, to the best of our ability to determine, healthy young adults. It was impossible to control the past history of exposure to respiratory diseases or chronic irritation of the respiratory tract for most of the animals used as trachea donors. However, the similarity of our findings among all the animals tested, including chronic respiratory disease-free rats and specific pathogen-free guinea pigs, argues against there being a major error introduced into our results by cryptic intercurrent lung or airways disease in any of the animal species tested.

With most of the animals examined (chicken, mouse, humans, guinea pig, presumably also sheep and pig), there seemed to be additional acidic components (probably proteoglycans) being secreted by tracheae into the medium; these additional components (Fig. 3) were minor when compared with the amount of labelled glycoprotein present in all species, and accounted for more than a few per cent of the total radioactivity released into the culture medium only with guinea pigs and pigs.

Tracheobronchial mucus is a heterogeneous mixture of complex macromolecular components. The great similarity in qualitative and quantitative indices of mucus glycoprotein synthesis and secretion by tracheobronchial organ explants from a wide variety of experimental animals and from humans is striking. Such a similarity lends confidence to extrapolation of these types of data, obtained upon exposure of animals to various pneumotoxins and/or air pollutants under rigorously controlled conditions, to potentially important health effects in human beings exposed to the same noxious agents [for example, Last & Cross (1978)].

These results also suggest that studies of the effects of air pollutants on tracheobronchial clearance mechanisms and upon other parameters that may be related to mucus glycoprotein production may be directly compared among the diverse species of experimental animals used in such studies. Especially noteworthy in this respect is that studies of tracheobronchial clearance in rats (Melville & Iravani, 1975), cats (Laurenzi *et al.*, 1968), guinea pigs (Drazen & Austen, 1975), non-human primates (Lucas & Douglas, 1934) and chickens (Battista & Kensler, 1970) have been reported by different research groups. Investigations such as the biochemical analysis of variations in tracheobronchial secretions and measurements of tracheobronchial clearance in experimental animals exposed to toxic inhalants are in their infancy and have not as yet resulted in many practical applications. Further refinements of such experimental designs may serve as models that will permit the assessment of environmental interactions with respiratory epithelium, including the role of such interactions in augmenting or compromising the integrated mucociliary clearance apparatus and the possible long-term sequelae of such interactions.

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